The Metabolism and Antiketogenic Effects of Sorbitol. Sorbitol Dehydrogenase

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Using the perfused liver of fasted or phlorrhizinized dogs, Embden & Griesbach (1914) discovered that sorbitol was converted into fermentable reducing sugars from which the only identifiable osazone prepared was phenylglucosazone. On the basis of changes in optical rotation and in the Seliwanoff reaction during perfusion, these authors considered that the primary product of sorbitol oxidation was fructose which was subsequently transformed into glucose. Additional evidence consistent with this view was provided by Anschel (1930), who observed a large increase in the excretion of fructose following administration of sorbitol to a case of fructosuria.

The work of Embden & Griesbach prompted the suggestion that sorbitol might prove a useful carbohydrate source in diabetes mellitus (Thannhauser & Meyer, 1929); but attempts to assess the utilization of sorbitol in diabetics by measurement of respiratory quotients and blood-sugar levels led to much controversy. The evidence has been reviewed by Carr & Krantz (1945). The effect of feeding sorbitol upon the deposition of glycogen in rat liver has also been the subject of conflicting reports. Nevertheless, most workers agree that sorbitol can act as a glycogen precursor (Carr & Forman, 1939; Johnston & Deuel, 1943; Blatherwick, Bradshaw, Ewing, Larson & Sawyer, 1940). Some insight into the enzymic mechanism of these metabolic reactions was gained by Breusch (1942, 1943), who demonstrated sorbitol dehydrogenase activity in brei prepared from the livers of starved cats. The criteria employed were the reduction of oxaloacetate and of methylene blue.

The work described in this paper was undertaken with the object of obtaining more precise definition of the initial steps in sorbitol metabolism. Such information is particularly desirable in view of the antiketogenic property of sorbitol, described by Edson (1936), who found that sorbitol is more effective than glucose or fructose in suppressing spontaneous ketogenesis in the liver slices of starved rats.

EXPERIMENTAL

Rats of the Wistar strain, aged 8–12 months, were used. They were fed a stock diet of pollard, bran, maize meal, meat meal and bone flour. In starvation experiments food was completely withheld for 22–36 hr. but free access to water allowed.

Tissue slices were cut by the conventional freehand method. Approx. 15 mg. dry weight of slices per Warburg flask were used.

Homogenates were prepared with the all-glass Potter-Elvehjem apparatus. The organs were removed immediately after the animals had been killed by a blow on the head or, in the case of rats, by rupturing the spinal cord, and chilled in ice water. After homogenizing, the material was kept at 0° until used.

Manometric methods. The standard methods of Warburg were used for the measurement of O_2 consumption by slices, homogenates and other preparations. Slices were suspended in phosphate saline, pH 7·4 (Krebs, 1933). Substrates dissolved in a small volume of distilled water were added to the main compartment of Warburg vessels; in controls with substrate omitted an equal volume of distilled water was added. Acid substrates were added as neutral Na salts; amines were added as neutralized hydrochlorides. Flasks were equilibrated in the thermostat for 7–10 min. before manometric readings were commenced.

Analytical methods

Sorbitol was determined by the periodate method of Rappoport, Reifer & Weinmann (1937).

Reducing sugar was determined according to Somogyi (1945*a*) after deproteinizing with $Ba(OH)_2$-$ZnSO_4$ (Somogyi, 1945*b*). When experiments were performed in the presence of methylene blue, the dye was removed from the deproteinized filtrate by treatment with a minimum amount of Merck's medicinal charcoal. Reducing sugar was determined in the solution (Somogyi, 1945*a*) after removal of the charcoal by filtration.

Ketose was determined by Cole's modification of Roe's method (see Bacon & Bell, 1948). When necessary the solution was cleared of methylene blue as above. The colour intensity was estimated by measuring optical density at 483 mμ. In estimations of fructose or sorbose the colour was always compared with that developed in a standard solution of the hexose in question, because sorbose gives approximately half the colour of an equal concentration of fructose.

Glucose was determined manometrically using glucose oxidase (Keilin & Hartree, 1948) in the presence of catalase (solution of crystallized enzyme), but without the addition of ethanol which raised the blank O_2 uptake to an unduly large and variable value.

Glucose oxidase was prepared from *Aspergillus niger* (National Collection of Type Cultures, no. 594), a culture of which was kindly supplied by Dr J. S. D. Bacon. The mould was grown as described by Mann (1944), harvested on the 4th day of incubation, and a dry powder prepared from the mycelium by homogenizing in a Waring blender with cold acetone. The acetone-dried powder was extracted with

0·03 M-potassium phosphate buffer, pH 6, the yellow filtrate treated with 7 vol. of cold acetone and the mixture immediately centrifuged in order to separate the viscous precipitate. The vacuum-dried precipitate was stable at 2°. When required, it was dissolved in 0·2 M-potassium phosphate buffer, pH 6, and dialysed against 0·01 M-potassium phosphate buffer, pH 6. This solution had high glucose oxidase activity, but did not oxidize galactose, fructose, sorbose, mannose or sorbitol.

Acetoacetic acid was determined by the aniline citrate method (Edson, 1935) except when determined simultaneously with β-hydroxybutyric acid when the modified Van Slyke technique (Edson, 1935) was employed.

Lactic acid was determined by the method of Friedemann & Graeser (1933).

Nitrogen was determined by the titrimetric micro-Kjeldahl procedure of Hiller, Plazin & Van Slyke (1948).

Coenzyme I (CoI) was estimated by the method of LePage (1947), using the value $E^{1\%}_{1\,cm.} = 8500$ at 340 mμ. In enzymic reactions the reduced coenzyme was estimated from the increase in optical density at 340 mμ. above the value of the blank at zero time.

Hexokinase activity was estimated according to Colowick & Kalckar (1943), aldolase activity according to Taylor, Green & Cori (1948).

pH *Measurements* were made with a glass electrode.

Optical density was measured in a Beckman quartz spectrophotometer, DU model.

Materials

Sorbitol. A sample of D-sorbitol monohydrate obtained from the Pfanstiehl Chemical Co. was used throughout this work. $[\alpha]_D^{23°} = -1·73 \pm 0·05°$ in water (*c*, 9·1).

Acetoacetate was prepared as described by Ljunggren (1924).

Cytochrome c was prepared by the method of Keilin & Hartree (1937) and assayed according to Potter (1945).

Coenzyme I. A crude preparation (12% purity) was made from baker's yeast by the method of Williamson & Green (1940). Some of this material was treated with norit according to Clark, Dounce & Stotz (1949), and the purity raised to 25%. Cozymase (purity 34 and 60%) obtained from Schwartz Laboratories, Inc., New York, was used in some experiments.

Reduced coenzyme I ($CoIH_2$) was prepared by the method of Ohlmeyer (1938).

Metabolic units

$-Q_{O_2}$ = μl. O_2 consumed/mg. dry wt. of tissue/hr.

Q_{Keto} = μl. CO_2 equivalent to β-ketonic acid formed/mg. dry wt. of tissue/hr.; 1 μmol. β-ketonic acid ≡ 22·4 μl. CO_2.

$Q_{Hydroxy}$ = μl. CO_2 equivalent to β-hydroxybutyric acid formed/mg. dry wt. of tissue/hr.; 1 μmol. β-hydroxybutyric acid ≡ 22·4 μl. CO_2.

RESULTS

Oxidation and antiketogenic effect of sorbitol in liver slices

Sorbitol is rapidly oxidized by rat-liver slices whether the animals have been well fed or starved. This is indicated by the increase in oxygen consumption observed when sorbitol is present in the suspension medium (Table 1). In agreement with Edson (1936) it was consistently found that 0·01 M-sorbitol caused a marked decrease (25–50%) in acetoacetate formed endogenously by liver slices from fasted rats. Since this observation does not exclude the possibility of acetoacetate being reduced to β-hydroxybutyrate in the presence of sorbitol, both ketone bodies were determined in presence and absence of sorbitol. The results show that formation of β-hydroxybutyrate was decreased to about the

Table 1. *Oxidation and antiketogenesis in rat-liver slices*

(Slices incubated in conical Warburg vessels containing 3·00 ml. of phosphate saline, pH 7·4. Inseals contained 0·20 ml. 2 N-NaOH. Time, 2 hr. Gas, O_2. Temp. 38°.)

Exp. no.	State of animal	Substrate added (0·01 M)	$-Q_{O_2}$	Q_{Keto}
1	Well fed	Nil	11·9	1·02
		Pentane-1-carboxylate	16·1	3·78
		Pentane-1-carboxylate + sorbitol	17·0	4·22
2	Fasted 36 hr.	Nil	11·0	1·93
		Sorbitol	15·8	0·83
		Glucose	11·7	1·73
		Fructose	13·9	1·29
		Sorbose	9·8	1·04
		L-Arabinose	11·7	1·62
		D-Arabinose	11·8	1·41
3	Fasted 36 hr.	Nil	13·8	3·82
		Glucose-1-phosphate (K salt)	13·5	3·33
4	Fasted 24 hr.	Nil	10·4	2·33
		Sorbitol	12·7	0·89
		Malonate (0·01 M)	10·3	2·68
		Sorbitol + malonate (0·01 M)	9·6	1·47
5	Fasted 24 hr.	Nil	11·8	3·44
		Sorbitol	15·4	1·89

In Exp. 5, $Q_{Hydroxy}$ was 0·96 in absence of added substrate, 0·50 in presence of sorbitol.

same extent as acetoacetate production in the presence of 0·01 M-sorbitol (Table 1, Exp. 5). Thus sorbitol decreased the total ketone bodies formed spontaneously in liver slices of starving rats.

Although sorbitol reduces the spontaneous ketogenesis of liver slices considerably, this compound has little effect on the production of ketone bodies during oxidation of pentane-1-carboxylic acid added to liver slices (Table 1). None of the carbohydrates tested exerted an effect on spontaneous ketogenesis as great as that of sorbitol (Table 1). Likely products of sorbitol oxidation (glucose, fructose and sorbose) possessed weaker antiketogenic properties when tested in a concentration of 0·01 M. The fact that neither L- nor D-arabinose exerted an appreciable antiketogenic effect has special interest, since Breusch (1943) reported that cat liver exhibits dehydrogenase activity for D-arabinose.

The antiketogenic effect of sorbitol is capable of several interpretations: it could operate (1) by inhibiting the oxidation of ketone-body precursors such as fatty acids, pyruvate and certain amino-acids, (2) by diverting the oxidation of fatty acids and pyruvate through pathways which do not lead to acetoacetate, or (3) by oxidative removal of the ketone bodies formed. There is evidence to indicate that in the liver complete oxidation via the Krebs tricarboxylic acid cycle is the main alternative to ketone-body formation (Lehninger, 1946; Grafflin & Green, 1948). Consequently, the effect of Krebs-cycle inhibitors, such as malonate, on antiketogenesis due to sorbitol possesses some significance.

The effect of malonate was tested by incubating liver slices from fasted rats with malonate in presence and absence of sorbitol. The results indicate that 0·01 M-malonate caused an increase in the amount of acetoacetate formed both in presence and absence of sorbitol, but by no means abolished the antiketogenic action (Table 1). Consequently this experiment affords no evidence that the Krebs cycle is directly concerned in the antiketogenic effect of sorbitol.

Products of the oxidation of sorbitol by liver slices

During the oxidation of sorbitol by liver slices of fasted rats, there appeared in the medium an extra amount of reducing sugar (calculated as glucose) approximately equivalent to the sorbitol disappearing; but when liver slices of well fed rats were used the extra reducing sugar formed was only slightly in excess of that appearing in the control without sorbitol (Table 2).

In experiments on a larger scale sorbitol was oxidized in presence of liver slices, and the suspension fluid analysed for total reducing sugar, glucose (using glucose oxidase) and ketose. Table 3 shows that sorbitol was largely converted to glucose, but a small amount of ketose was invariably produced. The amount of lactic acid formed was very small (0·07 mg./ml. of suspension medium). The

Table 2. *Formation of reducing sugar during sorbitol oxidation by rat-liver slices*

(Conditions as in Table 1.)

Exp. no.	State of animal	Substrate	$-Q_{O_2}$	Reducing sugar (calc. as glucose) (μmol./mg. dry wt.)	Sorbitol disappearing (μmol./mg. dry wt.)
1	Well fed	Nil	10·8	1·28	0
		Sorbitol (0·01 M)	14·8	1·33	0·66
2	Well fed	Nil	11·5	1·00	0
		Sorbitol (0·02 M)	10·6	1·24	0·72
3	Fasted 22 hr.	Nil	12·9	0·09	0
		Sorbitol (0·01 M)	15·2	0·66	0·68
4	Fasted 24 hr.	Nil	11·2	0·07	—
		Sorbitol (0·01 M)	12·1	0·50	—

Table 3. *Products of the oxidation of sorbitol by liver slices*

(Slices from male rats, fasted 36 hr., suspended in 12·0 ml. of phosphate saline, pH 7·4, in 150 ml. Erlenmeyer flasks. Sorbitol concentration, 0·01 M. Time, 4 hr. Gas, O_2. Temp. 38°. Suspension fluid deproteinized with $Ba(OH)_2$-$ZnSO_4$ before determination of optical rotation and analysis. For determination of reducing sugar, glucose and ketose see Analytical methods section.)

Rat no.	Dry wt. of slices (mg.)	Sorbitol added (mg.)	$\alpha_D(l, 4)$	Reducing sugar (calc. as glucose) (mg.)	Glucose (mg.)	Ketose (calc. as fructose) (mg.)
1	104·8	Nil	—	4·0	—	—
	109·3	21·6	+0·05°	19·0	18·0	0·24
2	102·9	Nil	—	2·4	—	—
	119·0	21·6	+0·05°	18·0	16·5	0·24
3	93·3	Nil	—	3·8	—	—
	123·0	21·6	+0·05°	20·8	17·7	0·26

values of the optical rotation are consistent with the observed concentrations of reducing sugars. The reducing material reacted with phenylhydrazine under the conditions specified by Edson (1940) to give a crystalline compound with the typical appearance of phenylglucosazone accompanied by a small amount of amorphous material. The reducing sugar was completely fermented by a suspension of washed baker's yeast. In experiments similar to those in Table 3 it was found that no ketose was formed in absence of sorbitol, and the deproteinized filtrate had an optical rotation of $-0{\cdot}01 \pm 0{\cdot}01°$ (l, 4).

Embden & Griesbach (1914) obtained evidence that during the oxidation of sorbitol by perfused dog liver, fructose was the primary product, but was subsequently converted to glucose. Confirmation of such a sequence was sought in rat liver, sorbitol being incubated with slices for varying periods of time, and the suspension medium analysed for ketose and total reducing sugar (Table 4). Although a significant concentration of ketose was found in all cases, no initially high proportion of ketose could be detected, the ketose concentration rising parallel to that of total reducing sugar.

Table 4. *Production of ketose during sorbitol oxidation by liver slices*

(Rat fasted 36 hr. Slices incubated in conical Warburg vessels in 3·10 ml. phosphate saline, pH 7·4. Inseals contained 0·20 ml. 2N-NaOH. Gas, O_2. Temp. 38°.)

Sorbitol (0·01 M)	Time (hr.)	Reducing sugar (calc. as glucose) (μg./mg. dry wt.)	Ketose (calc. as fructose) (μg./mg. dry wt.)
Absent	0·5	5·0	0·00
Present		44·0	2·20
Absent	1·0	5·5	0·00
Present		74·5	4·35
Absent	1·5	3·5	0·00
Present		111·5	3·25
Absent	2·0	0·5	0·00
Present		153·0	5·85
Absent	2·5	10·0	0·00
Present		167·0	6·75

Oxidation of sorbitol by homogenates of rat liver

Homogenates of rat liver, prepared in water or in 0·01 M-potassium phosphate buffer, pH 7·6, exhibited an increased oxygen uptake in the presence of sorbitol. Oxidation of sorbitol by homogenates required the presence of Co I and was considerably enhanced by the addition of nicotinamide and cytochrome *c*. With such a preparation, in which the cytochrome system was responsible for electron transfer to molecular oxygen, addition of potassium chloride also caused a slight increase of oxygen uptake in presence of sorbitol. The optimum concentration for each of these components is shown in Table 5.

In order to determine the intracellular localization of the enzyme oxidizing sorbitol, rat liver was homogenized in ice-cold 0·88 M-sucrose. All subsequent operations were carried out in the cold room (2°). Nuclei, whole cells and debris were removed by centrifuging at 600*g* for 10 min. The supernatant (fraction 1) was decanted and recentrifuged twice in the same way. In order to remove mitochondria, 0·11 vol. of 1·5 M-sodium chloride was added and the mixture kept for 5 min. in an ice bath. The agglutinated mitochondria were centrifuged at 2400*g* for 10 min., and the sediment (fraction 2) resuspended in 0·15 M-sodium chloride. The whole homogenate and the several fractions were then tested for their ability to oxidize sorbitol by measuring oxygen consumption (Table 6).

Most of the active material remained in the supernatant (fraction 1) obtained after removal of nuclei. Neither the mitochondria (fraction 2) nor the final supernatant (fraction 3) consumed much oxygen, but recombination of supernatant and mitochondria restored sorbitol oxidation to the initial level. The most probable explanation of this result is that a sorbitol dehydrogenase is located chiefly in fraction 3 (Table 6), which is composed of submicroscopic particles and soluble material. Since cytochrome oxidase and other insoluble components of the cytochrome system are largely confined to the mitochondria (Hogeboom, Claude & Hotchkiss, 1946), the system which causes oxygen uptake in the presence of sorbitol is not complete until the mitochondria and an adequate concentration of cytochrome *c* have been added. This hypothesis is supported by determinations of oxygen uptake in which methylene blue was substituted for the cytochrome system. Under these conditions fraction 3 showed a high oxygen uptake in the presence of sorbitol without addition of the mitochondrial fraction. In all subsequent experiments methylene blue was used to link the sorbitol dehydrogenase system to molecular oxygen.

The ability of whole homogenates to oxidize sorbitol decreased rapidly even at 0°, but the final

Table 5. *Optimum concentrations of components required for oxidation of sorbitol by rat-liver homogenates*

(Enzyme: 0·50 ml. of a 10% water homogenate of rat liver, centrifuged at 400 *g* for 10 min. to remove cell debris and nuclei. In each series the concentration of a single added component was varied, the concentrations of all other added components being maintained at the optimum level indicated by an asterisk. Total vol., 3·00 ml. Inseals contained 0·20 ml. 2N-NaOH. Time, 20 min. Gas, air. Temp. 38°.)

Component varied ...	Nicotinamide		CoI (12% purity)		Cytochrome *c*	
	Concn. (M)	O_2 uptake (μl.)	Concn. ($\times 10^{-4}$ M)	O_2 uptake (μl.)	Concn. ($\times 10^{-4}$ M)	O_2 uptake (μl.)
	0·0000	38	0·00	7	0·000	24
	0·0017	59	0·30	24	0·161	40
	0·0033	59	0·60	41	0·322	45
	0·0067	65	0·90	44	0·644*	53
	0·0100*	64	1·20*	46	0·966	47
	0·0150	62	1·80	53	1·449	46

Component varied ...	Sorbitol		KCl		Potassium phosphate, pH 7·6	
	Concn. (M)	O_2 uptake (μl.)	Concn. (M)	O_2 uptake (μl.)	Concn. (M)	O_2 uptake (μl.)
	0·0000	15	0·000	35	0·0000	50
	0·0033	36	0·033	36	0·0017	47
	0·0067	42	0·067	42	0·0033	45
	0·0100	43	0·100*	42	0·0050	55
	0·0133*	51	0·150	40	0·0067*	51
	0·0267	52	0·200	48	0·0100	51

* Concentration selected as optimum.

Table 6. *Distribution of sorbitol dehydrogenase in fractions of rat-liver homogenate*

(Enzyme: 0·50 ml. of homogenate or homogenate fraction. Each Warburg vessel contained in addition 0·30 ml. of KCl (0·10 M)*, 0·20 ml. potassium phosphate, pH 7·6 (0·067 M), 0·30 ml. nicotinamide (0·01 M), 0·20 ml. cytochrome *c* ($0{\cdot}644 \times 10^{-4}$ M), 0·20 ml. sorbitol (0·013 M, replaced by water in controls), 0·20 ml. CoI (12% purity; $1{\cdot}20 \times 10^{-4}$ M) and water to give a final vol. of 3·00 ml. Inseals contained 0·20 ml. 2N-NaOH. Time, 1 hr. Gas, air. Temp. 38°.)

Homogenate fraction	Sorbitol	O_2 uptake (μl.)	Extra O_2 uptake (μl.)
Whole homogenate	Absent	53	93
	Present	146	
Fraction 1: supernatant from nuclei	Absent	55	79
	Present	134	
Fraction 2: suspension of mitochondria	Absent	30	12
	Present	42	
Fraction 3: supernatant from mitochondria	Absent	35	29
	Present	64	
Fraction 2 + fraction 3	Absent	38	78
	Present	116	

* The figures in parentheses indicate the concentration of the components in the complete reaction medium. This notation is used in Tables 6–17 and Figs. 1–6.

supernatant from the homogenate (fraction 3) retains its activity unchanged for 24 hr. at 5°, and loses only 20% during incubation for 1 hr. at 38° (pH 7·8).

Extraction and partial purification of sorbitol dehydrogenase

The livers of three or four rats were rapidly removed, chilled in ice water and freed from remnants of diaphragm and mesentery. After cooling for several minutes the livers were quickly blotted dry, weighed and homogenized for 1·5–2 min. in 3 vol. of ice-cold 0·01 M-potassium phosphate buffer, pH 7·8, by means of a previously chilled Waring blender. The extract was rapidly cooled to 0° by vigorous stirring in a beaker placed in ethanol at −30°. In subsequent steps the temperature of the extract was kept between 0 and 5°. The pH was next adjusted to 4·7 by dropwise addition of 2N-HCl with vigorous stirring. After centrifuging in the cold room (2°) at 1100 *g* for 15 min. a red, slightly turbid supernatant (1, Table 7) of high activity was obtained. Several methods were used to fractionate this extract.

Preparation A. In earlier experiments the extract was adjusted to pH 4·0 by dropwise addition of 0·4N-HCl, and then to pH 7·8 by dropwise addition of 5% (w/v) KOH.

Table 7. *Purification of sorbitol dehydrogenase*

(Enzyme preparation, 0·25 or 0·50 ml., adjusted to pH 7·8 and tested as soon as prepared. Flasks contained in addition 0·25 ml. sorbitol (0·017 M, replaced by water in controls), 0·50 ml. potassium phosphate, pH 7·9 (0·033 M) and water to give a final vol. of 3·00 ml. Methylene blue (0·20 ml.; 0·001 M) and 0·50 ml. Co I (12% purity, $3·0 \times 10^{-4}$ M) were added from the side arm after equilibration. Inseals contained 0·20 ml. 2 N-NaOH. Gas, air. Temp. 38°. Values of O_2 uptake based on first 10 min. period, and corrected for uptakes in absence of substrate.)

Exp. no.	Enzyme preparation	Volume (ml.)	O_2 uptake (μl./hr.) Per mg. N	Per mg. dry wt.	Per ml. of solution	Total
1	Whole homogenate	130·8	—	6·4	456	59,400
	Whole homogenate adjusted to pH 4·7; ppt. discarded. Supernatant 1	95	—	11·3	300	29,400
	Supernatant 1 adjusted to pH 4·0 and then to pH 7·8. Ppt. discarded. Supernatant 2	84	—	10·1	240	22,200
	Supernatant 2 treated with ethanol-$CHCl_3$. Ppt. discarded. Supernatant 3	90	—	43·1	168	17,400
2	Supernatant 1	80	171	20·5	396	31,800
	Supernatant 2	83	154	17·3	300	24,900
	Supernatant 3	117	422	51·8	228	26,400
	Supernatant 3 twice fractionated with $(NH_4)_2SO_4$; dialysed	25	—	50·0	300	7,800

There was no precipitate at pH 4, but after this treatment a precipitate appeared on readjustment of the pH to 7·8. All subsequent steps were performed in the cold room at 2°. After removal of the precipitate (discarded) by centrifugation at 1100 *g* for 10 min., solid $(NH_4)_2SO_4$ was added to the supernatant (2, Table 7) to give a concentration of 2·15 M. The precipitate which formed was centrifuged down and discarded, and the concentration of $(NH_4)_2SO_4$ in the supernatant brought to 3·2 M by addition of the solid.

After centrifuging again at 1100 *g* for 30 min., the supernatant (discarded) was decanted carefully in order to conserve the light precipitate, which was dissolved in the minimum volume of 0·01 M-potassium phosphate, pH 7·6. After thorough dialysis against distilled water at 2°, and removal by centrifugation of the small precipitate which sometimes formed, the solution had an O_2 uptake of 240 μl. O_2/mg. N/hr. when tested under the conditions described in Table 8. The solution, which was coloured by haemoglobin and a little cytochrome *c*, had a very low O_2 uptake in absence of substrate.

Preparation B. Later it was found that a preparation of greater activity could be obtained by the use of ethanol and $CHCl_3$. After preliminary adjustment of the pH of the extract first to 4·0 and then to 7·8, followed by centrifuging to remove the precipitate which formed, the supernatant (2, Table 7) was mixed with 0·25 vol. of 95% (v/v) ethanol and 0·25 vol. of $CHCl_3$ added. The mixture was shaken vigorously in a stoppered vessel at room temperature for 1 min. and immediately returned to the cold room for centrifugation at 1100 *g* for 15–30 min. The denatured haemoglobin, cytochrome and other proteins formed a solid layer at the $CHCl_3$-water interface from which the supernatant aqueous layer was pipetted. The solution was thoroughly dialysed against distilled water at 2° to give an almost clear, yellowish-brown solution (3) the O_2 uptake of which under standard conditions (Table 7) was 420 μl. O_2/mg. N/hr. This preparation also possessed negligible O_2 uptake in absence of substrate.

Attempts to increase the activity of preparation *B* by fractionation with ammonium sulphate or ethanol were unsuccessful, although by this means coloured substances were separated from the active material. From Table 7 it may be seen that adjustment of the original extract to pH 4·0 and then to 7·8, followed by centrifugation, caused no increase in activity. However, when this step was omitted from the procedure, the activity of the final preparation was appreciably lower.

Oxidation of D-*sorbitol by dehydrogenase preparations*

Enzyme preparations made by the above procedures oxidize D-sorbitol rapidly in the presence of Co I, either aerobically using methylene blue for hydrogen transport to molecular oxygen, or anaerobically using methylene blue as hydrogen acceptor. Table 8 shows the anaerobic oxidation of sorbitol by the enzyme, and Figs. 1 and 2 show the effect of varying the concentration of methylene blue and Co I, respectively, on the oxygen uptake in aerobic experiments. Since Co II was not available, it was not possible to test the coenzyme specificity of the dehydrogenase.

Table 8. *Anaerobic oxidation of sorbitol by the dehydrogenase*

(Thunberg tubes contained 0·25 ml. Co I (12% purity, $1·5 \times 10^{-4}$ M), 0·25 ml. sorbitol (0·017 M), 1·00 ml. potassium phosphate, pH 8·0 (0·067 M), 0·20 ml. of indicator (approx. 10^{-4} M), and water to give a final vol. of 3·00 ml. Enzyme (0·50 ml. of preparation *B*) added from stopper after evacuation. Temp. 38°.)

	Indicator	Reduction time
Complete system	Methylene blue	18 min.
Co I omitted	Methylene blue	>5 hr.
Sorbitol omitted	Methylene blue	>5 hr.
Complete system	Janus green	Partial reduction (rose)
Complete system	Benzyl viologen	Partial reduction

Soluble diaphorase prepared from pig heart according to Straub (1939) caused a 27 % increase in oxygen consumption of the enzyme (preparation *B*)

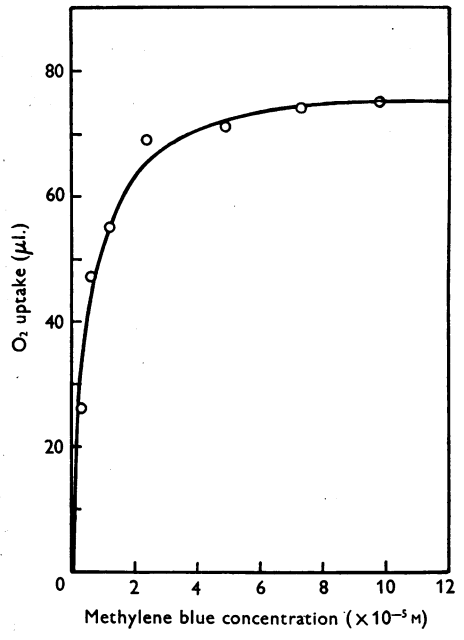


Fig. 1. Effect of methylene blue concentration on sorbitol oxidation. Flasks contained 0·30 ml. enzyme (preparation *A*), 0·25 ml. sorbitol (0·017 M), 1·00 ml. potassium phosphate, pH 8·0 (0·067 M), 0·25 ml. Co I (34 % purity, $0·85 \times 10^{-4}$ M), methylene blue (added from side arm after equilibration) and water to give a final vol. of 3·00 ml. Gas, air. Temp. 38°. Time, 15 min.

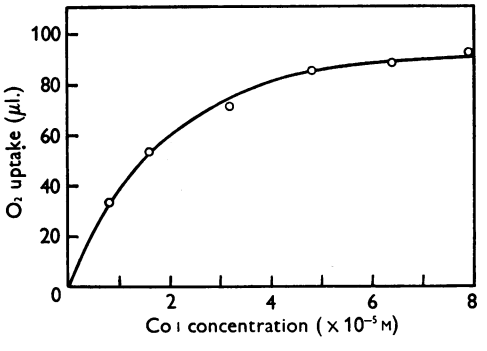


Fig. 2. Effect of Co I concentration on sorbitol oxidation. Conditions as in Fig. 1. Methylene blue concentration 10^{-3} M.

when methylene blue and Co I were present in optimum concentrations. This indicates that as in other dehydrogenase systems, diaphorase accelerates the rate of oxidation with methylene blue.

Effect of temperature. This is shown in Fig. 3. The optimum temperature under the particular conditions was 38–40°, and the activity declined steeply outside this range.

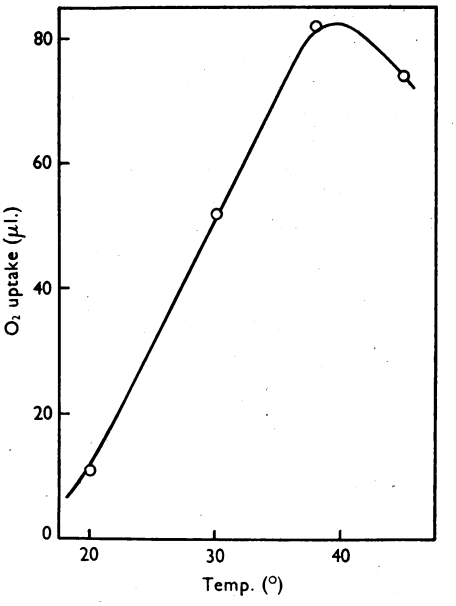


Fig. 3. Effect of temperature on sorbitol dehydrogenase activity. Conditions as in Fig. 1. Methylene blue concentration 10^{-3} M. Time, 30 min.

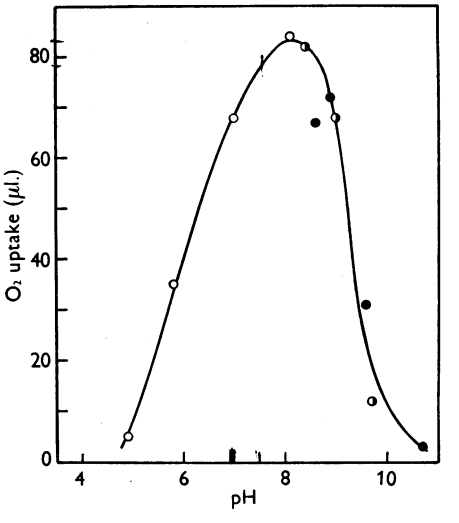


Fig. 4. Effect of pH on sorbitol dehydrogenase activity. Conditions as in Fig. 1 except that various buffers (0·067 M) were used. Time, 30 min. ○—○, Phosphate; ◑—◑, glycine-NaCl; ●—●, veronal.

Effect of pH. Fig. 4 illustrates the relationship of pH to enzymic activity, the optimum range being pH 7·9–8·1. This was confirmed by measuring the rate of Co I H_2 formation during oxidation of sorbitol

in absence of methylene blue. The enzyme appears to lose little activity within the pH range 5–9 when incubated at 38° for 40 min. in absence of substrate. At pH 3·2 activity is completely destroyed in a few seconds at 0°.

Enzyme-substrate affinity. From data on the influence of sorbitol concentration on the initial rate of oxygen uptake by the enzyme at 38° in presence of phosphate buffer (pH 8·0) and optimum amounts of Co I and methylene blue, the Michaelis constant (K_m) was found by the method of Lineweaver & Burk (1934) to be 7×10^{-4} M.

Absorption spectrum. The spectrum of the purest enzyme preparations showed a peak at about 280 mμ., but no other bands of intense absorption. Since no band appeared in the vicinity of 340 mμ. on addition of sorbitol, it may be concluded that no Co I is bound to the dehydrogenase.

Substrate specificity. The enzyme is highly specific for D-sorbitol and L-iditol (see below). Many other compounds containing alcohol groups were tested, but glyceraldehyde was the only one oxidized by the most highly purified preparations of the enzyme. In the purest preparations DL-glyceraldehyde was oxidized at 12% of the rate (determined spectrophotometrically) found for D-sorbitol.

In manometric experiments enzyme (preparation *A*) supplied with methylene blue and Co I did not oxidize L-malate, pyruvate, DL-lactate, glucose or fructose. Examined spectrophotometrically, purified preparations (*A*) displayed no activity towards the following: D-mannitol, dulcitol, inositol, L-sorbose, D-fructose, D-glucose, D-gluconate, L-ascorbic acid; DL-lactate, pyruvate, dihydroxyacetone, DL-β-hydroxybutyrate; α- and β-glycerophosphate, glycerol, glycerate, glycollate; choline, ethanolamine, DL-serine, DL-threonine; glycolaldehyde diethylacetal, ethylene glycol, ethanol, *n*-propanol, *n*-butanol, and *n*-pentan-1-ol. Substrates were tested at concentration of 0·01 M, except the last six which were tested at 0·002 M.

Preparations of high activity possessed negligible aldolase, hexokinase or fructokinase activity, and only slight phosphatase activity when tested at pH 6·0 (succinate buffer) and 9·0 (veronal buffer) with β-glycerophosphate as substrate.

Inhibitors. Table 9 shows the effect of various inhibitors on sorbitol dehydrogenase. The action of boric acid may be due either to formation of a weakly dissociated complex with sorbitol, or to true inhibition of the enzyme. In spectrophotometric experiments it was found that 0·028 M-boric acid, pH 8·0, largely inhibited both the oxidation of sorbitol and the reduction of fructose and sorbose. Since sorbose has no pair of adjacent *cis*-hydroxyl groups, it is unlikely to form a complex with boric acid, and true inhibition of the enzyme is more probable in this case.

Table 9. *Effect of inhibitors on sorbitol dehydrogenase*

(Flasks contained 0·50 ml. enzyme (preparation *A*), 0·25 ml. sorbitol (0·016 M), 0·25 ml. Co I (12% purity, $1{\cdot}4 \times 10^{-4}$ M), 1·00 ml. potassium phosphate, pH 8·0 (0·063 M), inhibitor and water to give a final volume of 3·20 ml. Methylene blue (0·2 ml.; $9{\cdot}8 \times 10^{-4}$ M) was added from the side arm after equilibration. Gas, air. Time, 20 min. Temp. 38°.)

Inhibitor	O_2 uptake (μl.)	Inhibition (%)
Nil	215	—
Sodium borate, pH 7·8 (0·02 M)	10	95
Sodium iodoacetate (0·01 M)	148	31
Urethane (0·20 M)	206	4
NaN_3 (0·02 M)	230	−7
Mannitol (0·02 M)	203	5
Dulcitol (0·02 M)	208	3
Malonate (0·01 M)	210	2
n-Octanol (saturated solution)	198	8
KCN, pH 8 (0·01 M)	104	53
Na_2S, pH 8 (0·01 M)	228	−6

Distribution. Sorbitol dehydrogenase activity in other tissues of the rat and in the livers of various species was estimated by the increase in oxygen uptake of homogenates due to addition of sorbitol under the conditions shown in Table 10. In the first series of experiments the tissues were homogenized in 4 vol. of ice-cold 0·01 M-potassium phosphate, pH 8·0, adjusted to pH 5·0, centrifuged and the supernatant used for activity determination. The extract was kept at 0–2° until used. Since the loss of activity incurred in preparing this extract varied greatly in different tissues, the whole homogenate was used in a second series of experiments. With this procedure there is the disadvantage of more rapid loss of activity during both the equilibration and experimental periods. The two procedures gave results (Table 10) which differ significantly for rat kidney and for cat and rabbit liver, but which are otherwise parallel. Although only semi-quantitative, they show that sorbitol dehydrogenase is confined to liver and kidney in the rat, and is widely distributed in the livers of other species.

Reversibility of sorbitol oxidation

The time course of sorbitol oxidation was followed by measuring the rate of $Co\,I H_2$ formation when sorbitol and Co I reacted in the presence of a buffered sorbitol-dehydrogenase preparation. The reaction proceeded at exactly the same rate in the presence of phosphate as of glycylglycine buffer of similar pH and concentration.

When D-fructose was added to the reaction mixture after it had approached equilibrium, there was an immediate and progressive decline in $Co\,I H_2$ concentration until a second equilibrium state was reached. L-Sorbose, when added instead of fructose, also reoxidized $Co\,I H_2$ but at a slower rate (Fig. 5). D-Glucose had no effect. These experiments suggest

Table 10. *Distribution of sorbitol dehydrogenase activity*

(Flasks contained 0·50 or 1·00 ml. of whole homogenate or extract, 0·25 ml. sorbitol (0·017 M, replaced by water in controls), 1·00 ml. potassium phosphate, pH 8·0 (0·067 M), 0·25 ml. CoI (12% purity, 3×10^{-4} M), 0·20 ml. methylene blue (0·001 M) and water to give a final volume of 3·00 ml. With whole homogenate, flasks contained 0·30 ml. nicotinamide (0·01 M) and CoI was added from the side arm after equilibration; with extract, methylene blue was added from the side arm after equilibration. Inseals contained 0·20 ml. 2 N-NaOH. Gas, air. Temp. 38°.)

	O_2 uptake (μl./ml. homogenate or extract/initial 10 min.)					
	Extract			Homogenate		
Tissue	Sorbitol	Control	Difference	Sorbitol	Control	Difference
Rat liver	51	19	32	90	52	38
Rat kidney	10	8	2	60	24	36
Rat spleen	9	8	1	12	10	2
Rat heart	8	6	2	26	24	2
Rat lung	7	6	1	6	8	0
Rat skeletal muscle	8·5	6·5	2	6	8	0
Rat brain	7	5	2	0	2	0
Rat testis	12	7	5	4	0	4
Cat liver	13	17	—	103	44	59
Mouse liver	39	13	26	148	62	86
Guinea pig liver	47	16	31	115	41	74
Albino rabbit liver	9	10	0	105	37	68
Wild rabbit liver	31	20	11	140	70	70
Frog liver	11·5	7·5	4	55	40	15

that D-sorbitol is reversibly oxidized to L-sorbose and D-fructose in presence of sorbitol dehydrogenase and CoI. Since glucose was unable to reoxidize $CoIH_2$, it is presumably not a product of sorbitol oxidation. Glucose-6-phosphate and fructose-6-phosphate were also inactive with the enzyme when tested by this method, and neither glucose nor glucose-6-phosphate was converted to fructose or fructose-6-phosphate when incubated with the buffered enzyme and reduced CoI, either with or without adenosinetriphosphate and magnesium ions.

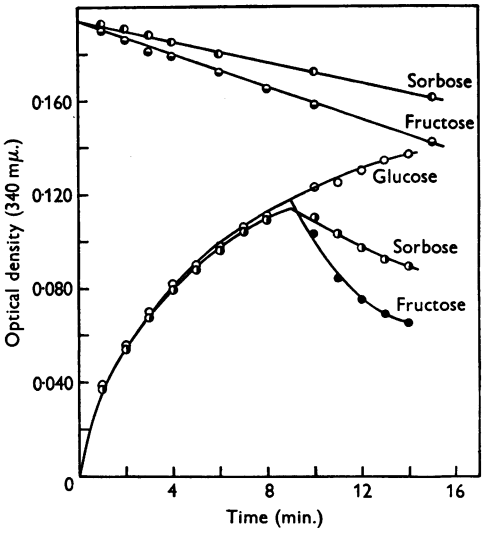


Fig. 5. Reversibility of sorbitol oxidation. Tubes contained 0·50 ml. enzyme (preparation *B*), 0·50 ml. potassium phosphate, pH 7·9 (0·033 M), substrate, CoI or $CoIH_2$ (3×10^{-5} M) and water to give a final volume of 3·00 ml. ◐—◐, $CoIH_2$ and L-sorbose (0·038 M). ◒—◒, $CoIH_2$ and D-fructose (0·038 M). ○—○, CoI and D-sorbitol (0·0033 M); after 9 min. 0·05 ml. D-glucose (0·0041 M) added. ●—● CoI and D-sorbitol (0·0033 M); after 9 min. 0·05 ml. D-fructose (0·0041 M) added. ◑—◑, CoI and D-sorbitol (0·0033 M); after 9 min. 0·05 ml. L-sorbose (0·0041 M) added.

The initial rate of enzymic oxidation of $CoIH_2$ (used as sodium salt, 20% purity) by L-sorbose was 69% of the rate obtained with D-fructose (Fig. 5). The ketoses did not react with $CoIH_2$ in absence of enzyme.

Products of the oxidation of sorbitol by the dehydrogenase

The above experiments suggest that D-fructose and L-sorbose, but not D-glucose, are formed during the enzymic oxidation of D-sorbitol. The question of glucose formation was investigated by oxidizing sorbitol enzymically under aerobic conditions in the presence of CoI and methylene blue, and testing the deproteinized solution, freed of methylene blue, for glucose with glucose oxidase. The results showed the absence of glucose. The solution, however, gave a strong test for ketose and it was possible to demonstrate (Table 11) that the ratios, oxygen uptake : ketose formed (determined colorimetrically and calculated as fructose) : sorbitol utilized, were in accord, within the limits of experimental error, with the stoicheiometric requirements of the equation

$$C_6H_{14}O_6 + \tfrac{1}{2}O_2 \rightarrow C_6H_{12}O_6 + H_2O.$$

Because of the difficulty of estimating sorbitol by periodate in the presence of large amounts of hexose, incubation was continued in Exps. 1–6

Table 11. *Oxygen uptake, ketose formation and sorbitol disappearance*

(Flasks 1–6 contained 1·25 ml. enzyme (preparation *B*), flasks 7–11 contained 1·00 ml. In addition flasks contained 0·25 ml. Co I (12% purity, 2×10^{-4} M), 0·50 ml. phosphate, pH 8·0 (0·033 M), sorbitol and water to give a final vol. of 3·20 ml. Flasks 7–11 also contained 0·50 ml. of a diaphorase preparation. Methylene blue (0·2 ml.; $9{\cdot}4 \times 10^{-4}$ M) added from side arm after equilibration. Exps. 1–6 continued till O_2 uptake ceased. Exps. 7–11, time, 2·25 hr. O_2 uptake corrected for that in absence of substrate (16 μl./hr.). Gas, air. Temp. 38°.)

Exp. no.	Initial sorbitol concn. ($\times 10^{-6}$ M)	Sorbitol disappearing (μmol.)	O_2 uptake (μatom)	Ketose formed (calc. as fructose) (μmol.)
1	0·61	2·0	2·0	—
2	1·56	5·0	5·4	—
3	3·13	10·0	10·1	—
4	4·54	15·0	15·4	—
5	6·05	20·0	19·2	—
6	9·07	30·0	27·2	—
7	1·26	—	4·7	4·2
8	2·52	—	7·0	7·1
9	3·16	—	7·9	8·2
10	3·79	—	9·6	9·1
11	4·38	—	9·7	10·2

(Table 11) until oxygen uptake ceased. On the assumption that all the sorbitol had been oxidized, the amount disappearing was taken as equal to the amount initially added. The agreement obtained (Table 11) with the theoretical values justifies the assumption made for the purpose of calculating ketose concentration, that the ketose was indeed fructose. Under the conditions of the experiment no carbon dioxide is formed during oxidation. In larger-scale experiments (Table 12) it was confirmed that fructose was the chief product of the enzymic oxidation of sorbitol.

Table 12. *Fructose formation from sorbitol*

(Enzyme (93 ml., preparation *B*) incubated with sorbitol (0·048 M) and Co I (12% purity, $9{\cdot}6 \times 10^{-5}$ M), dissolved in 5·0 ml. potassium phosphate, pH 7·9 ($9{\cdot}6 \times 10^{-3}$ M) and 6·0 ml. methylene blue ($9{\cdot}5 \times 10^{-4}$ M). Mixture (104 ml.) divided among six 150 ml. Erlenmeyer flasks. Time, 14 hr. Gas, O_2. Temp. 38°. Optical rotation measured and analyses performed on deproteinized filtrate, freed of methylene blue. Controls without substrate had zero rotation, and contained no reducing sugar or ketose.)

	Concn. (mg./ml.)
Total reducing sugar	3·02
Fructose (calc. from $\alpha_D = 1{\cdot}06 \pm 0{\cdot}01°$ in water; *l*, 4)	2·87
Ketose (calc. as fructose from colorimetric determination)	2·66

The formation of fructose was demonstrated by the preparation of osazones. A solution of reaction products prepared by the method outlined in Table 13 (4 ml.) gave characteristic crystals of phenylglucosazone. In another experiment the entire deproteinized solution, freed of methylene blue, was evaporated to dryness under reduced pressure (bath temperature 40°), the residue extracted with boiling 95% ethanol, and the extract filtered and evaporated to dryness under reduced pressure. The residue was taken up in 1·5 ml. of α-methylphenylhydrazine sulphate reagent (Neuberg & Mandl, 1946) and incubated at 25° for 1 hr. As controls 200 mg. of D-glucose and 200 mg. of D-fructose were treated separately with the reagent in the same way. Crystallization of α-methylphenylfructosazone could not be induced within this period at 25° as reported by Neuberg & Mandl (1946), and in other experiments only crude crystalline material was obtained after several days at 2°. After incubation at 25° the osazone was therefore extracted according to Bacon & Bell (1948), and recrystallized successively from chloroform-light petroleum, 20% (v/v) ethanol, ethyl acetate and finally ethyl acetate-light petroleum (b.p. 40–60°). The last mixture proved to be the best solvent for crystallization, readily giving fine yellow to orange needles, free from contaminating amorphous material which was otherwise difficult to remove. Observed microscopically on a melting-point block the crystals melted at 151–153° (decomp.), while the sample prepared simultaneously from authentic D-fructose had m.p. 152·5–153·5° (decomp.). In capillary tubes heated in a bath these samples had m.p.'s 130–132° (decomp.) and 133–135° (decomp.) respectively, and gave a mixed m.p. 134–137° (decomp.). Further recrystallization did not raise the melting point. Glucose gave no crystalline product.

While this establishes fructose as the main product of the enzymic oxidation of sorbitol there remains the possibility that a smaller quantity of L-sorbose is formed. The enzymic reduction of sorbose by Co I H_2 seems to indicate that this may be the case. Sorbose, however, could not be detected in experiments in which the accumulated products of aerobic sorbitol oxidation were fermented with baker's yeast and subsequently tested for ketose.

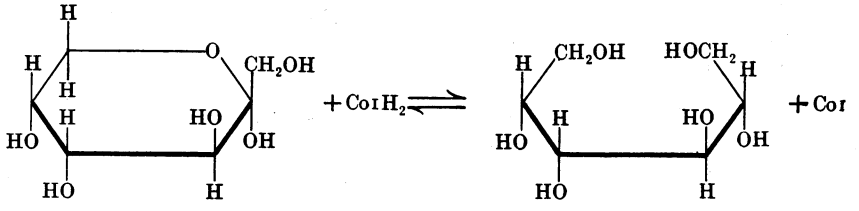
Table 13 *Enzymic reduction of sorbose and fructose*

(Enzyme (2·00 ml., preparation *B*) incubated with 0·50 ml. hexose (0·02 M), 0·20 ml. Co I H_2 (20% purity, 1×10^{-4} M) and 0·30 ml. potassium phosphate, pH 7·9 (0·02 M). Time, 3 hr. Temp. 38°. Deproteinized with $Ba(OH)_2$-$ZnSO_4$, and 3·00 ml. of filtrate used for sorbose determination after fermenting with 0·5 vol. of a 10% (w/v) suspension of washed baker's yeast in potassium phosphate, pH 6·0 (0·20 M) for 6 hr. at 25°.)

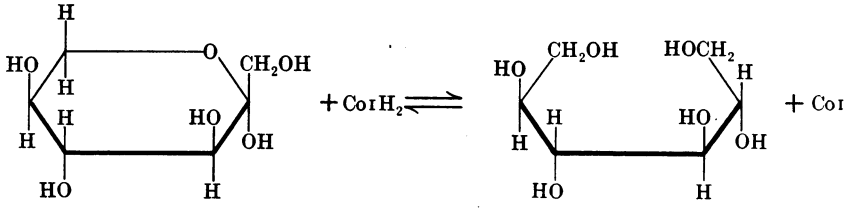
	Initial substrate (0·02 M)	After incubation: Fermentable ketose (μg.)	After incubation: Unfermented ketose (μg.)
Complete system	Fructose	640	48
	Sorbose	0	840
Co I H_2 omitted	Fructose	806	37
	Sorbose	0	1043
Heat-inactivated enzyme	Fructose	902	42
	Sorbose	0	992

An attempt to accumulate sorbose more rapidly was made by incubating sorbitol with enzyme and Co I in the presence of fructose. Under these conditions conversion of fructose to sorbitol should maintain a steady rate of sorbose formation by keeping the concentration of sorbitol and Co I constant. No sorbose could be detected in the deproteinized solution. The sensitivity of the method is such as to permit the conclusion that if sorbose is formed by enzymic oxidation of sorbitol it constitutes less than 1% of the total ketose produced.

An attempt to demonstrate the interconversion of L-sorbose and D-fructose through D-sorbitol as an intermediate was made by incubating each of the hexoses with buffered sorbitol dehydrogenase and



α-D-Fructopyranose D-Sorbitol



L-Sorbopyranose L-Iditol

Co I H_2 (Table 13). Although an appreciable disappearance of each hexose occurred, no increase in unfermentable ketose above that in the control with heat-inactivated enzyme was detected in the presence of fructose, and no fermentable sugar was produced from sorbose. The small amount of unfermented 'ketose-reacting' substance present both in controls and in experiments with fructose was shown to be derived from the yeast.

Oxidation and antiketogenic effect of L-*iditol*

The absence of detectable quantities of sorbose amongst the products of sorbitol oxidation, in spite of the reduction of L-sorbose by Co I H_2 in the presence of the dehydrogenase, suggests that sorbose may be reduced to some other hexitol. When the pyranose structures of D-fructose and L-sorbose are compared it may be seen that the reversible reduction of D-fructose to D-sorbitol corresponds stereochemically to the reversible reduction of L-sorbose to L-iditol.

It would have been impossible to test this hypothesis without the kindly assistance of Prof. G. Bertrand, who generously presented crystalline specimens of D- and L-iditol (synthetic) and L-iditol (natural 'sorbierite'). It was found that L-iditol

(natural and synthetic) was readily oxidized by rat-liver slices (Table 14) and by CoI in presence of the dehydrogenase preparation (Fig. 6). On the other hand, D-iditol was not oxidized under the same conditions, and the enzyme apparently has little affinity for this compound since D-iditol, like D-mannitol and dulcitol, does not competitively inhibit the oxidation of D-sorbitol or L-iditol by the dehydrogenase.

Table 14. *Oxidation and antiketogenic effect of* L-*iditol*

(Liver slices from a rat fasted 24 hr. were incubated in conical Warburg vessels containing 3·00 ml. of phosphate saline, pH 7·4. Inseals contained 0·20 ml. 2N-NaOH. Time, 2 hr. Gas, O_2. Temp. 38°.)

Substrate (0·01 M)	$-Q_{O_2}$	Q_{Keto}
Nil	10·7	2·78
Sorbitol	14·3	0·97
L-Iditol ('sorbierite')	12·8	0·88
L-Iditol (synthetic)	11·9	0·82
D-Iditol (synthetic)	11·1	2·94

It was of considerable interest to determine whether L-iditol, being readily oxidized by rat liver, could depress the spontaneous ketogenesis of liver slices from starved rats, or whether the antiketogenic effect was specific to sorbitol. It was found that L-iditol is effectively antiketogenic to the same extent as sorbitol, whilst D-iditol, like mannitol and dulcitol, has no effect (Table 14).

Products of iditol oxidation

The oxidation of L-iditol by the coenzyme-dehydrogenase system produced a non-fermenting ketose. When the concentration of ketose was calculated on the assumption that the colour developed was due to sorbose, agreement was obtained between the amounts of $CoIH_2$ and sorbose formed in the oxidation of L-iditol (Table 15). Although the ketose has not been isolated, it is almost certainly L-sorbose.

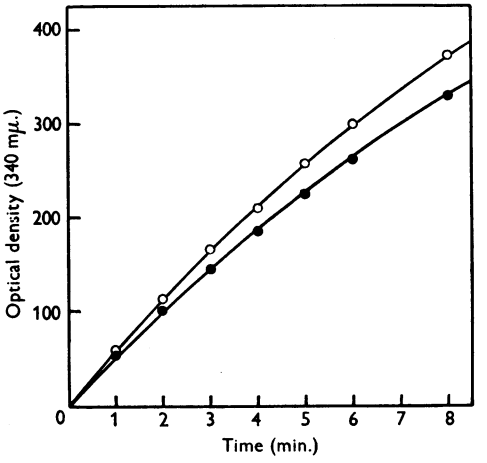


Fig. 6. Oxidation of L-iditol. Tubes contained 0·50 ml. enzyme (preparation *A*), 2·15 ml. potassium phosphate, pH 7·8 (0·0073 M) and 0·20 ml. CoI (60% purity, 0·0075 M). Hexitol (0·01 ml.; 0·0063 M) added at zero time. O—O, D-Sorbitol; ●—●, L-iditol.

The oxidation of L-iditol by rat-liver slices produced glucose and a non-fermenting ketose, probably sorbose (Table 16). Compared with the products of sorbitol oxidation by slices, much less glucose and much more ketose accumulated.

Table 15. *Products of the reaction of* L-*iditol with* CoI

(Tubes contained 0·50 ml. dehydrogenase (preparation *B*), 2·15 ml. potassium phosphate, pH 7·8 (0·0073 M), 0·20 ml. CoI (60% purity, 0·0075 M) and 0·10 ml. hexitol (0·0063 M). Temp. 20°. Time, about 3 hr.)

	Reaction products		
Hexitol added	$CoIH_2$ (μmol.)	Total ketose (μmol.)	Unfermentable ketose (μmol.)
Sorbitol	0·74	0·73*	0·00
L-Iditol ('sorbierite')	0·76	0·85†	0·79†
L-Iditol (synthetic)	0·74	0·80†	0·79†
D-Iditol (synthetic)	0·00	0·00	—

* Calculated as fructose. † Calculated as sorbose.

Table 16. *Products of the oxidation of* L-*iditol by rat-liver slices*

(Slices from a rat fasted 24 hr., suspended in 12·0 ml. of phosphate saline, pH 7·4, in 150 ml. Erlenmeyer flasks. Iditol concentration 0·01 M. Time, 4 hr. Gas, O_2. Temp. 38°. Suspension fluid deproteinized with $Ba(OH)_2$-$ZnSO_4$. For determination of reducing sugar, glucose and ketose see Analytical methods section.)

Dry wt. of slices (mg.)	Iditol added (mg.)	Reducing sugar (calc. as glucose) (mg.)	Glucose (mg.)	Ketose (calc. as sorbose) (mg.)
103·3	Nil	1·0	0·6	0·0
98·6	21·6	6·8	5·7	2·7

Equilibrium constant for sorbitol-fructose reaction

The equilibrium constant for the reversible oxidation of sorbitol to D-fructose can be calculated from the concentrations of sorbitol, fructose, CoI and $CoIH_2$ at equilibrium. By making the concentrations of sorbitol and fructose high compared with those of coenzyme, the former can be regarded as remaining at their initial levels. Hence the equilibrium constant can be obtained from the values of the initial sorbitol and fructose concentrations and the determined CoI and $CoIH_2$ concentrations. $CoIH_2$ was estimated by the spectrophotometric method, and CoI was calculated by difference from total assayed coenzyme concentration.

In a preliminary experiment the total coenzyme concentration was calculated from the dilution of a stock coenzyme solution, assayed by the dithionite method (LePage, 1947). From a series of eight reaction mixtures with varying ratios of sorbitol to fructose, the mean value of the equilibrium constant was found to be 0·230. In the second experiment (Table 17) the total coenzyme concentration was determined by enzymic reduction with 0·118M-sorbitol, since at equilibrium the $CoIH_2$ may be equated to the total coenzyme present without appreciable error.

reduction indicators (Table 8). From the above data ΔF° at 20° for the reaction

D-Sorbitol (aq.) $\rightarrow$ D-fructose (aq.) $+ 2H^+ + 2e$

is $8{\cdot}5 \times 10^3$ cal./mol. at pH 8·0.

DISCUSSION

The demonstration of the existence of sorbitol dehydrogenase reacting with CoI provides an explanation for the oxidation of sorbitol by perfused dog liver (Embden & Griesbach, 1914), cat-liver brei (Breusch, 1942, 1943) and rat-liver slices and homogenates. The presence of dehydrogenase activity in liver homogenates from all mammalian species examined suggests that the enzyme has widespread occurrence.

It has been established with partially purified dehydrogenase preparations from rat liver that the product of sorbitol oxidation is D-fructose, which is probably metabolized further in homogenates and intact cells via the glycolytic sequence of reactions. It has been shown also that the dehydrogenase oxidizes L-iditol, the evidence indicating that the product is L-sorbose. In the presence of buffered dehydrogenase preparations and coenzyme the oxidation both of L-iditol and of sorbitol is reversible (Figs. 5 and 6), and the equilibrium constant for the latter reaction has been determined (Table 17). It is

Table 17. *Equilibrium constant for reversible oxidation of sorbitol*

(Tubes contained 0·59 ml. enzyme (preparation *B*), 0·49 ml. potassium phosphate, pH 8·0 (0·03M), 0·32 ml. CoI (12% purity, $1{\cdot}2 \times 10^{-4}$M), sorbitol, fructose and water to give a final vol. of 3·40 ml. Temp. 20·0±0·5°. Time, approx. 1·5 hr., until $E_{340\,m\mu}$ was constant.)

Concentrations of reactants				
Sorbitol ($\times 10^{-2}$M)	Fructose ($\times 10^{-2}$M)	CoI ($\times 10^{-5}$M)	$CoIH_2$ ($\times 10^{-5}$M)	K
10·59	0·72	2·64	9·65	0·249
10·00	1·09	3·79	8·50	0·244
9·41	1·45	4·62	7·67	0·256
8·82	1·81	5·50	6·79	0·253
8·23	2·17	6·52	5·77	0·233
7·65	2·52	7·03	5·26	0·246
6·47	3·26	8·43	3·86	0·231
5·29	3·98	9·48	2·81	0·223
4·12	4·70	10·19	2·10	0·235
2·94	5·43	10·96	1·33	0·224

The data for the second determination shown in Table 18 give $K\,(20°) = 0{\cdot}240$ with a 95% confidence interval of $\pm 0{\cdot}013$. This corresponds to $\Delta F^\circ = 832$ cal. in the oxidation of sorbitol by CoI. Taking E_0 at 30° $= -0{\cdot}072$ V. and

$$\Delta E / \Delta T = -0{\cdot}00043 \text{ V.}$$

for $CoI/CoIH_2$ (Borsook, 1940), E_0 for the system sorbitol/fructose at 20° is $+0{\cdot}185 - (0{\cdot}058 \times \text{pH})$ V. $= -0{\cdot}283$ V. at pH 8·0.

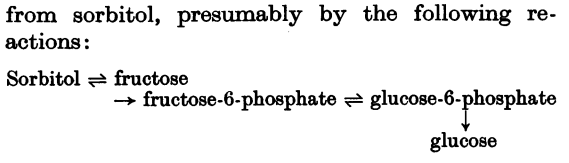
This value for E'_0 at pH 8 is in good agreement with the behaviour of the system with oxidation-reasonable to assume that the equilibrium constant for the iditol-sorbose reaction has a similar value.

The dehydrogenase is highly specific, D-sorbitol and L-iditol being the only substrates reacting significantly. The slow oxidation of glyceraldehyde by liver extracts is probably due to another component of the enzyme preparation since the rate of oxidation of this substrate was very low in the purest preparations of the enzyme. Since D-glucose and D-gluconate are not oxidized, the enzyme requires the presence of a primary alcohol group at C-1. Furthermore, the inability of the enzyme to oxidize D-

mannitol and dulcitol indicates that the configuration of the substrate at C-2 and C-4 must conform to the pattern in D-sorbitol for reaction to occur. No direct evidence has been obtained regarding the configuration at C-3, the only substrate tested which differed from D-sorbitol in its configuration at this centre (D-iditol) differing also in the configuration at C-2 and C-4.

In the above discussion it has been assumed that a single enzyme is responsible for the sorbitol-fructose and iditol-sorbose reactions, but it is possible that different dehydrogenases are involved. If the enzymes are not identical it is likely that the reactions are catalysed by dehydrogenases of the same type which would be CoI-linked.

Liver slices of fasted rats produce mainly glucose from sorbitol, presumably by the following reactions:



The extent to which a glucose-sparing effect of sorbitol contributes to the accumulation of glucose could not be assessed with the techniques employed. Only small amounts of fructose can be detected in the suspension medium (Table 3). Assuming that the accumulated glucose is derived directly from sorbitol, it may be concluded that in liver slices of fasted rats oxidation of sorbitol to fructose and conversion of fructose to glucose proceed much more rapidly than hexose oxidation.

The physico-chemical characteristics of the enzymic oxidation of sorbitol afford no obvious explanation of the marked antiketogenic action (Tables 1 and 14). The substrate-enzyme affinity ($K_m = 7 \times 10^{-4}$ M), the equilibrium constant (K, 0·240) and the corresponding redox potential (E'_0 at pH 8·0 = −0·283 V.) are of the same order as those of several other dehydrogenases. However, the fact that both D-sorbitol and L-iditol are antiketogenic despite the formation of different oxidation products, suggests that the dehydrogenation itself is the source of the antiketogenesis. Two other effectively antiketogenic compounds, glycerol and glyceraldehyde, may well owe their effect also to rapid dehydrogenation. The actual mechanism by which the dehydrogenation interacts with fatty acid oxidation must at present remain a matter for speculation.

SUMMARY

1. The oxidation of D-sorbitol in rat-liver slices and homogenates is catalysed by a dehydrogenase using coenzyme I. The dehydrogenase also oxidizes L-iditol; the specificity is discussed.

2. The product of the oxidation of D-sorbitol by the partially purified dehydrogenase is D-fructose. L-Iditol is probably oxidized to L-sorbose.

3. The main product of the oxidation of sorbitol by liver slices is glucose. Very small quantities of fructose are also formed. Oxidation of L-iditol by slices produces small amounts of glucose and sorbose.

4. Sorbitol and iditol considerably lower the spontaneous ketogenesis of liver slices from fasted rats.

5. The oxidation of sorbitol by coenzyme I to fructose is reversible; the equilibrium constant is 0·240 ± 0·013. The oxidation of L-iditol to L-sorbose is also reversible.

6. The optimum conditions for the oxidation of sorbitol by the liver dehydrogenase are pH 7·9–8·1 and 38–40°. $K_m = 7 \times 10^{-4}$ M.

7. Sorbitol dehydrogenase is found in the rat only in liver and kidney. It appears to be widely distributed in mammalian liver.

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The Action of 2:4-Dinitrophenol on Oxidative Phosphorylation

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In a previous paper (Judah & Williams-Ashman, 1951) the effect of a number of compounds was tested on oxidative phosphorylation in cell-free systems. 2:4-Dinitrophenol (DNP) was selected for further study because of widespread interest in this compound and because its action was particularly clear-cut and reproducible.

The starting point of the present work was the investigation of the inhibition of pyruvate oxidation by DNP; this has led to an attempt at localization of the action of DNP and to a study of the 'replacement' of inorganic orthophosphate by DNP (Loomis & Lipmann, 1948) in systems deficient in phosphate. Some experiments on the action of azide and observations on the effect of thyroxine on oxidative phosphorylation are also included.

METHODS

All methods are as described previously (Judah & Williams-Ashman, 1951) with the following exceptions.

Enzyme preparations. Preparations of mitochondria from liver and kidney of rat and rabbit were invariably carried out by the method of Schneider (1948), using 0·25 M-sucrose as the medium. This method gives a uniform preparation free of cell debris, erythrocytes and cell-nuclei, which form a large part of such preparations as 'cyclophorase' (Green, Loomis & Auerbach, 1948). For homogenization, the apparatus of Potter & Elvehjem (1936) was used. A blender yielded suspensions which were far less active and which invariably failed to show proportionality between tissue concentration and respiratory rate. Furthermore, the yields of mitochondria were low when the tissue was disintegrated in the blender. All preparations were carried out at 0°; the mitochondrial suspensions obtained were found to be stable for several hours when kept cold; preparations stored for as long as 6 hr. showed no decline in the efficiency of oxidative phosphorylation. Considerable uniformity in properties was observed from one preparation to another (O_2 uptake with pyruvate being approx. 500 μl. O_2/mg. N/hr.), and no inactive preparations have been encountered in a series of several hundred.

The mitochondria tolerated repeated resuspension and sedimentation without appreciable loss of activity, particular preparations having been subjected to a cycle of five or six such operations. In washing the particles care was taken to ensure an even suspension in the sucrose medium. This was achieved by the use of a mechanically rotated pestle which fitted the centrifuge tube loosely. A few seconds sufficed to disperse the tightly packed particles, which were dispersed in six to eight 50 ml. cellulose tubes, washings being conducted with 25–30 ml. 0·25 M-sucrose per tube.

Microsomes (submicroscopic particles) were prepared as described by Schneider (1948), a force of 25,000 *g* being applied for 1 hr. to the suspension in 0·25 M-sucrose.

Reagents. Crystalline sodium or potassium pyruvate were prepared from commercial pyruvic acid after redistillation *in vacuo*. α-Ketoglutaric acid was obtained from Nutritional Biochemicals, Inc., Cleveland, Ohio. Inorganic pyrophosphate was an analytical reagent; on chromatography in ethyl acetate-pyridine (Hanes & Isherwood, 1949), it showed only a trace of inorganic ortho-P and otherwise gave a single homogeneous spot.

Estimations. Orthophosphate was estimated by Ba and Mg precipitation (Umbreit, Burris & Stauffer, 1949) and by Ca precipitation at neutral pH by an unpublished method of the writer. Phosphate balances were determined as described by LePage in Umbreit *et al.* (1949).

Nucleic acid estimations were carried out by the method of Schmidt & Thannhauser (1945).

Pyruvate and α-ketoglutarate were determined by the method of Friedemann & Haugen (1943), using either a Beckman Model DU spectrophotometer or a Unicam diffraction-grating instrument.

Ferricyanide was determined spectrophotometrically by its absorption at 420 mμ. after deproteinization with trichloroacetic acid.

N was determined by the micro-Kjeldahl method. Steam volatile acid was estimated by the method of Long (1938).